EXHIBIT 3

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Pteroylpolyglutamate Hydrolase from Human Jejunal Brush Borders

PURIFICATION AND CHARACTERIZATION*

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Pteroylpolyglutamate hydrolase was solubilized with Triton X-100 from human jejunal mucosal brush borders and purified approximately 5,000-fold using organomercurial affinity chromatography, DEAE-cellulose chromatography, and gel filtration. The apparent molecular weight of the purified enzyme in the Triton micelle was estimated as 700,000 using Bio-Gel A-1.5m gel filtration. Sodium dodecyl sulfate/urea-polyacrylamide gel electrophoresis followed by Coomassie stain demonstrated two polypeptide bands at 145,000 and 115,000 daltons. The purified enzyme had an isoelectric point of 7.2, was maximally active at pH 5.5, and was stable above pH 6.5 and at temperatures up to 65 °C for at least 90 min. Human jejunal brush-border pteroylpolyglutamate hydrolase is an exopeptidase which liberated [^{14}C]Glu as the sole labeled product of PteGlu$_2$[^{14}C]Glu (where PteGlu$_n$ represents pteroylpolyglutamate), failed to liberate a radioactive product from PteGlu$_2$[^{14}C]GluLeu$_2$, and released all possible labeled PteGlu$_n$ products during incubation with Pte[^{14}C]GluGlu$_6$ with the accumulation of Pte[^{14}C]Glu. PteGlu$_2$, PteGlu$_3$, and PteGlu$_7$ were substrates, each with K_m = 0.6 μM, whereas PteGlu was a weak inhibitor of the hydrolysis of PteGlu$_3$ with K_i = 20 μM. Components of the pteroyl moiety, Glu, and short chain Glu$_n$ in α or γ linkages were not inhibitory. The enzyme was activated by Zn^{2+} or Co^{2+}. The properties of brush-border pteroylpolyglutamate hydrolase are different from those described for the soluble intracellular pteroylpolyglutamate hydrolase in other species and in human mucosa, yet are consistent with previous data on the process of hydrolysis of PteGlu$_n$ in the intact human intestine.

During the process of intestinal absorption, pteroylpolyglutamates (PteGlu$_n$[1]), the predominant forms of dietary folates (1), are hydrolyzed to their pteroylpolyglutamate (PteGlu) derivatives, which are then transported across the jejunal mucosa (2). The hydrolytic enzyme pteroylpolyglutamate hydrolase has been found in human intestinal mucosa in several hundred-fold greater activity than in bile, pancreatic, or intraluminal fluids (3). Using human jejunal mucosa from patients undergoing elective jejunoileal bypass surgery, this laboratory demonstrated two separate intestinal mucosal pteroylpolyglutamate hydrolases with distinct pH optima, inhibition characteristics, and molecular size: one intracellular and the other located in the brush-border fraction (4). The presence of pteroylpolyglutamate hydrolase activity in human jejunal brush-border membranes can account for the recovery of products of Pte[^{14}C]GluGlu$_6$ hydrolysis in intestinal aspirates obtained during *in vivo* human jejunal perfusion of this compound (5). Thus, brush-border pteroylpolyglutamate hydrolase may play a principal role in the digestion and intestinal absorption of dietary folates. The objective of the present study was to purify and characterize pteroylpolyglutamate hydrolase in the brush-border membrane of human jejunal mucosa.

EXPERIMENTAL PROCEDURES[2]

RESULTS

Physical Characteristics—Table I summarizes the purification of brush-border pteroylpolyglutamate hydrolase from human jejunum. The presence of 0.1% Triton X-100 was required at each step to maintain enzyme solubilization. The Triton micelle containing pteroylpolyglutamate hydrolase eluted in a sharp peak from the Bio-Gel A-1.5m column at a position corresponding to an apparent molecular weight of 700,000. This contrasts with the previously reported molecular weight of 90,000 for this enzyme, which was based on a less pure enzyme preparation in which brush-border pteroylpolyglutamate hydrolase activity appeared near the void volume of a Sephadex G-200 column (4). Sodium dodecyl sulfate/urea-polyacrylamide gel electrophoresis of the Bio-Gel A-1.5m sample revealed two polypeptide bands after Coomassie Blue staining, with mobilities corresponding to 145,000 and 115,000 daltons (Fig. 1). An additional band appeared at 185,000 daltons with silver staining. Either the Bio-Gel A-1.5m fraction or the DEAE-cellulose column fraction was used for the kinetic studies reported in this paper.

Isoelectric focusing of brush-border pteroylpolyglutamate hydrolase yielded a pI of 7.2. The enzyme was stable at pH 6.5 and above for at least 90 min (Fig. 2*A*). Maximum activity of the purified enzyme occurred at pH 5.5 using a 15-min incubation in 3,3-dimethylglutarate buffer (Fig. 2*B*). This contrasts with the previously reported pH maximum of 6.5 using a less pure enzyme sample (14). Assays were routinely

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[1] The abbreviations used are: PteGlu$_n$, pteroylpolyglutamate; PteGlu, pteroylmonoglutamate.

[2] "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-1007, cite the authors, and include a check or money order for $2.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

TABLE I

Purification of brush-border pteroylpolyglutamate hydrolase

Fraction	Protein	Specific activity[a]	Purification factor	Yield
	mg	*milliunits/mg*		%
Mucosal homogenate[b]	6160	0.039	1	100
Triton X-100 solubilization of brush borders	124	0.63	16	32
Mercury-Sepharose	60.5	1.12	29	28
DEAE-cellulose	1.3	31.5	808	21
Bio-Gel A-1.5m	0.14	191.0	4900	10

[a] Expressed in milliunits of $Pte(Glu)_5$ hydrolyzed per mg of protein.
[b] 10% homogenate of 50 g of minced mucosa.

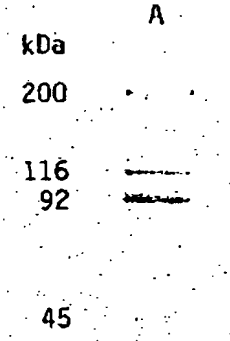

FIG. 1. **Sodium dodecyl sulfate/urea-polyacrylamide gel electrophoresis of brush-border pteroylpolyglutamate hydrolase.** *Lane A* (protein standards, 0.5 μg each) and *lane B* (6.0 μg of pteroylpolyglutamate hydrolase) were stained with 0.05% Coomassie Blue. Polypeptide bands were identified at 145,000 and 115,000 daltons, respectively.

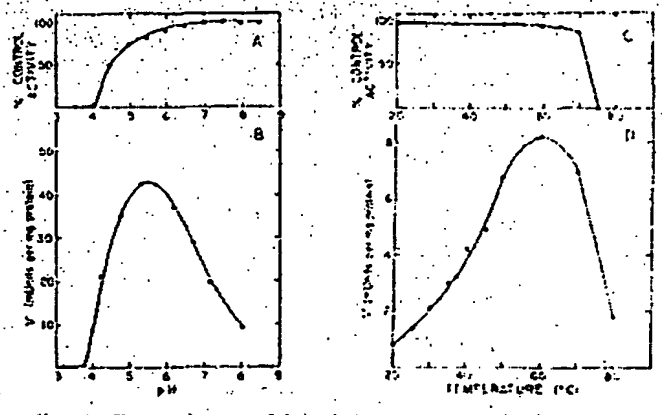

FIG. 2. **Dependence of brush-border pteroylpolyglutamate hydrolase on pH and temperature.** *A*, pH stability of brush border pteroylpolyglutamate hydrolase. Purified enzyme was preincubated at varied pH in 20 mM 3,3-dimethylglutarate buffer for 90 min. Samples were then assayed in standard reaction mixture at pH 6.5 for 15 min. Activity is plotted as per cent of nonpreincubated sample activity. *B*, pH-dependent activity profile of brush-border pteroylpolyglutamate hydrolase. Enzyme was assayed in standard reaction mixture using 33 mM 3,3-dimethylglutarate at varied pH for 15 min. *C*, temperature stability of brush-border pteroylpolyglutamate hydrolase. Enzyme was preincubated at varied temperatures in standard reaction mixture minus $Pte(Glu)_5$ for 90 min. After cooling on ice, enzyme activity was assayed at 37 °C as described under "Experimental Procedures." Activity is plotted as per cent of nonpreincubated sample activity. *D*, temperature-dependent activity profile of brush-border pteroylpolyglutamate hydrolase. Enzyme was assayed in 33 mM phosphate-buffered reaction mixture (pH 6.5) at varied temperatures for 15 min.

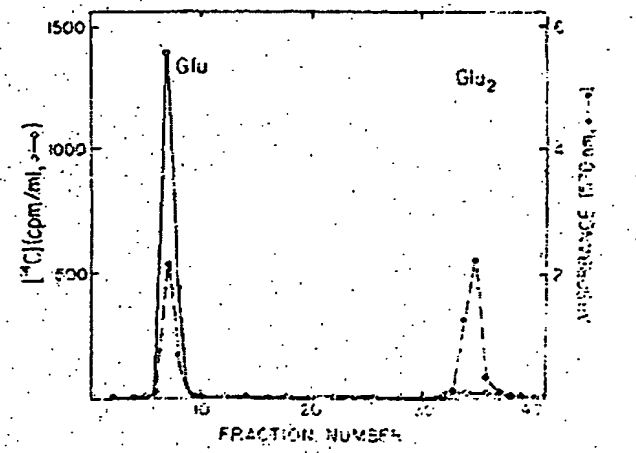

FIG. 3. **Glutamate reaction products of brush-border pteroylpolyglutamate hydrolase.** Partially purified enzyme was incubated at standard reaction mixtures for 30 min. The reaction was stopped with 3 ml of cold buffer containing 5 μM phosphate (pH 7.6), 0.1 mM $HgCl_2$, and 10 μmol of glutamic acid and 10 μmol of γ-glutamylglutamate as markers. The sample was applied to a 1 × 20 cm Bio-Rad AG 1-X8 column equilibrated with 5 mM phosphate buffer (pH 7.8). The column was eluted with a 0–0.2 M NaCl gradient (100-ml total volume), while collecting 5 ml fractions. The glutamates in even-numbered fractions were detected using ninhydrin reagent, and radioactivity was determined by scintillation counting.

performed at pH 6.5, since the enzyme was more stable at this pH. Pteroylpolyglutamate hydrolase was stable at temperatures up to 65 °C for at least 90 min (Fig. 2*C*). There was sharp drop in both stability and activity at temperatures greater than 70 °C. Maximal velocity was observed at 60 °C when the reaction time was 15 min (Fig. 2*D*).

Mechanism of Hydrolysis—The results from studies with three different substrates indicate that brush-border pteroylpolyglutamate hydrolase acts as an exopeptidase. As shown in Fig. 3, the carboxyl-terminal peptide bond was the initial cleavage site of $Pte(Glu)_2[^{14}C]Glu$, since [^{14}C]Glu was the sole labeled product. Brush-border pteroylpolyglutamate hydrolase failed to cleave the internal linkages of $Pte(Glu)_5[^{14}C]Glu_{Leu_2}$, as indicated by the absence of ^{14}C-labeled products. The $Pte(Glu)_n$ products of hydrolysis were investigated using $Pte[^{14}C]Glu(Glu)_6$ as substrate. As shown in Fig. 4, progressively shorter chain length labeled pteroylpolyglutamates appeared with longer incubation times with the accumulation of Pte[^{14}C]Glu, indicating that each compound was also a substrate for brush-border pteroylpolyglutamate hydrolase.

An initial velocity reciprocal plot with the most purified enzyme resulted in a K_m for $Pte(Glu)_5$ of 0.55 μM and a V_{max} of 200 nmol mg^{-1} min^{-1}. Fig. 5 shows reciprocal plots of the initial velocities when $Pte(Glu)_2$ was included in the reaction mixture. $Pte(Glu)_2$ was a competitive inhibitor of the reaction with a K_i of 0.6 μM obtained from the slope and intercept replots (Fig. 5 *inset*). $Pte(Glu)_4$ inhibition resulted in a similar reciprocal plot pattern and K_i (data not shown). Since PteGlu and $Pte(Glu)_2$ were alternate substrates of the enzyme (Fig. 4), their K_i values of $Pte(Glu)_n$ hydrolysis are equal to the K_m of the hydrolysis of each respective compound. PteGlu was also

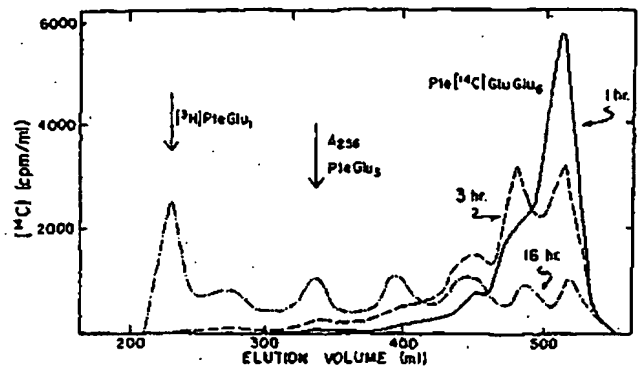

FIG. 4. **Pteroylglutamate reaction products of brush-border pteroylpolyglutamate hydrolase.** Partially purified enzyme was incubated in 12 ml of 22 μM Pte[^{14}C]GluGlu$_6$ and 50 μM zinc acetate with 20 mM phosphate buffer (pH 6.5). The reaction mixtures were incubated with 2 milliunits of enzyme for 1 h (——) and 3 h (– – –) and with 16 milliunits for 2 h (–·–·) (equivalent to 16 h of incubation with 2 milliunits of enzyme). Each reaction was terminated by dilution with 100 ml of cold 5 mM phosphate buffer (pH 7.0) containing 0.1 mM $HgCl_2$ and 0.5 μmol of PteGlu$_5$ and 0.4 μCi of [^{3}H]PteGlu as markers. The samples were then applied to 0.7 × 35-cm DEAE-cellulose columns equilibrated with 5 mM phosphate buffer (pH 7.0). The products were eluted with a 0.1–0.6 M NaCl gradient in 1-liter total volume. Radioactivity was detected using a scintillation counter.

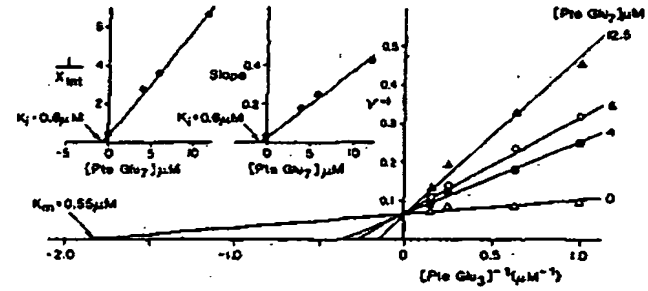

FIG. 5. **Competitive inhibition of PteGlu$_3$ hydrolysis by PteGlu$_7$.** The initial velocities at varied concentrations of PteGlu$_2$[^{14}C]Glu in the presence of different fixed concentrations of PteGlu$_7$ were determined in standard reaction mixtures. The reactions were started by the addition of enzyme. Data were plotted as initial velocity^{-1} *versus* [PteGlu$_3$]$^{-1}$. Slope is the slope of the primary plot lines, X_{int} is the x intercept of the primary plot.

TABLE II

Substrate affinities of brush-border pteroylpolyglutamate hydrolase

The K_m for PteGlu$_3$ was determined from a Lineweaver-Burk plot. The other values were determined from inhibition studies of PteGlu$_3$ hydrolysis.

Substrate	K_m	K_i
	μM	μM
PteGlu		20.0
PteGlu$_2$	0.6	0.6
PteGlu$_3$	0.55	
PteGlu$_7$	0.6	0.6

a competitive inhibitor of PteGlu$_3$ hydrolysis but had a K_i of 20 μM. These kinetic constants are summarized in Table II.

Studies of the effects of various PteGlu$_n$ moieties on pteroylpolyglutamate hydrolase activity suggested that the brush-border enzyme is a γ-carboxypeptidase which appeared to require the presence of the pteroyl terminus of the substrate. More than 95% inhibition of the standard reaction was seen with 0.1 mM PteGlu$_2$ or PteGlu$_7$. PteGlu at 0.1 mM caused 17% inhibition. Substituted and reduced PteGlu or *p*-aminobenzoylglutamate did not inhibit at 0.1 mM but caused 30% inhibition at 1.0 mM. Other components of the pteroyl moiety, including *p*-aminobenzoate, *N*-benzoylglutamate, *N*-*p*-nitrobenzoylglutamate, or pterin carboxylate and Glu or Glu$_n$ in γ or α linkage, were not inhibitory. Enzyme inhibition by pteroyl-linked compounds and lack of inhibition by Glu or Glu$_n$ suggest that an interaction of pteroylpolyglutamate hydrolase with the pteroyl terminus is required for subsequent cleavage of the γ-glutamyl linkages of the substrate. The requirement for the γ linkage is supported by the studies with PteGlu$_2$[^{14}C]GluLeu$_2$, in which the terminal α linkage of Leu$_2$ prevented cleavage and liberation of the ^{14}C label. Alternatively, a specific requirement of brush-border pteroylpolyglutamate hydrolase for terminal Glu could have prevented an exopeptidase reaction with this compound. However, others have shown that human liver pteroylpolyglutamate hydrolase is capable of cleavage of substituted peptides in γ linkage but not in α linkage (15).

The activity of purified brush-border pteroylpolyglutamate hydrolase was unaffected by the addition of *p*-hydroxymercuribenzoate at concentrations up to 0.5 mM. These data confirm a previous finding which used a less purified sample of the enzyme (4). There was no inhibition of pteroylpolyglutamate hydrolase activity in brush-border membrane fragments by cholate, deoxycholate, chenodeoxycholate, or their glycine or taurine conjugates at concentrations up to 1.0 mM. These latter data contrast with results of a previous study of the effect of bile acids which used a different assay method and mucosa containing both intracellular and brush-border pteroylpolyglutamate hydrolase (16).

Metal Ion Effects on Pteroylpolyglutamate Hydrolase—Dialysis against 1 mM phenanthroline and 1 mM EDTA abolished the activity of the enzyme. The addition of 100 μM Ni^{2+}, Fe^{2+}, Cd^{2+}, Ca^{2+}, Mn^{2+}, or Mg^{2+} caused slight activation, while Hg^{2+}, Pb^{2+}, or Cu^{2+} had no effect on pteroylpolyglutamate hydrolase activity. Assays performed with Zn^{2+} or Co^{2+} gave the same level of activation with respect to metal ion concentration, with 70% of the predialyzed enzyme activity restored at 20 μM of each metal. The activation of the enzyme by Zn^{2+} at different fixed concentrations of PteGlu$_3$ is shown in Fig. 6. Velocity plots were sigmoidal with respect to Zn^{2+} concen-

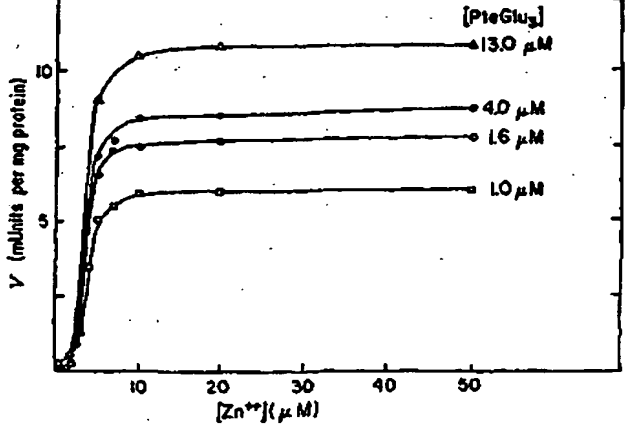

FIG. 6. **Activation of brush-border pteroylpolyglutamate hydrolase by Zn^{2+}.** The initial velocities at varied concentrations of Zn^{2+} and different fixed concentrations of substrate were determined in the presence of 0.13 M NaCl using the standard reaction mixture. Prior to the assays, the enzyme was treated with 1 mM EDTA and 1 mM phenanthroline, followed by dialysis to remove the chelators.

tration. At each concentration of substrate, there was a sharp increase in velocity at about 3 μM Zn^{2+}, and maximal velocity was achieved at 20 μM Zn^{2+}. Plots of v^{-1} *versus* $[Zn^{2+}]^{-1}$ were curved with asymptotes intersecting at about 3 μM Zn^{2+} (not shown). The sigmoidal velocity plot patterns suggest a cooperative activation (17) of brush-border pteroylpolyglutamate hydrolase by Zn^{2+} and show that this metal provides sensitive control over the velocity of the reaction.

Anion Effects—Extensive dialysis of pteroylpolyglutamate hydrolase against standard buffer minus zinc reduced the activity of the brush-border enzyme by 72%. Ninety-five per cent of predialyzed activity was recovered with the addition of 300 mM NaCl, KCl, or NaBr, and 85% of predialyzed activity was recovered with 300 mM $NaNO_3$ (Fig. 7). NaF inhibited the pteroylpolyglutamate hydrolase activity present in the dialyzed sample. The addition of sodium 3,3-dimethylglutarate to the reaction mixture at concentrations up to 200 mM had no effect on the activity of the dialyzed enzyme. These studies indicate that the activation of brush-border pteroylpolyglutamate hydrolase is not dependent on ionic strength and is increased in the presence of specific anions.

DISCUSSION

Pteroylpolyglutamate hydrolases have been identified in a variety of tissues where they function in the conversion of intracellular polyglutamyl folates to the monoglutamyl derivatives which appear in the circulation (18). Intestinal mucosal pteroylpolyglutamate hydrolase serves the specialized function of digesting dietary pteroylpolyglutamates before their absorption. The role of the enzyme in folate absorption and metabolism is poorly understood because of differences in enzyme properties among species and among different tissues within the same species. For example, pteroylpolyglutamate hydrolases purified with bovine (18) and human (15) liver or from rat (19) and chicken (20) intestine are all soluble enzymes which have greatest affinity for long chain polyglutamyl folates. Whereas both liver pteroylpolyglutamate hydrolases act as exopeptidases (15, 18), each intestinal enzyme has been reported to act as an endopeptidase producing either PteGlu (19) or $PteGlu_2$ (20) as the initial product of hydrolysis of $PteGlu_7$. Species differences in the properties of pteroylpolyglutamate hydrolase were further underscored by the identification of two enzyme activities in human jejunal mucosa, one bound to the brush-border membrane with a neutral pH optimum and the other a soluble intracellular enzyme with an acid pH optimum (4). Recent studies comparing intestinal pteroylpolyglutamate hydrolase activities in different species indicate that the brush-border enzyme is also present in pig but is absent in rat and monkey (21).

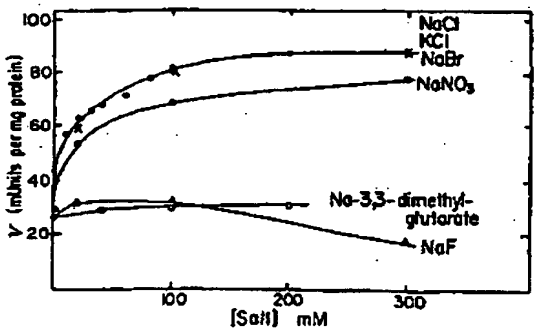

FIG. 7. **Effects of anions on brush-border pteroylpolyglutamate hydrolase.** The initial velocities at varied concentrations of salts were determined with standard reaction mixtures at 13 μM $PteGlu_2[^{14}C]Glu$. The enzyme was extensively dialyzed against standard buffer minus zinc prior to the assays.

The present studies indicate that human jejunal brush-border pteroylpolyglutamate hydrolase is an exopeptidase which is activated by zinc and is specific for pteroylpolyglutamates. The apparent molecular weight of 700,000 from gel filtration in the presence of Triton X-100 is considerably greater than that of 85,000 reported for soluble rat intestinal pteroylpolyglutamate hydrolase (19). Also in contrast to the rat jejunal enzyme, the human brush-border enzyme has equal affinity for $PteGlu_n$ of different chain lengths (Table II), requires the pteroyl moiety, and clearly acts as an exopeptidase. Recent data from our laboratory indicate that the soluble human jejunal intracellular pteroylpolyglutamate hydrolase is, by contrast, an endopeptidase that is capable of liberation of both $[^{14}C]Glu_2$ and $[^{14}C]Glu$ from $PteGlu_2[^{14}C]Glu$ and produces a ^{14}C-labeled compound from $PteGlu_2[^{14}C]GluLeu_2$ (22).

Correlation of results of the present *in vitro* studies of purified brush-border pteroylpolyglutamate hydrolase with data from previous clinical studies of the hydrolysis and absorption of $PteGlu_n$ suggests that this enzyme may play an essential role in the hydrolysis of dietary folates. Previously we showed that the jejunal perfusion of $Pte[^{14}C]GluGlu_6$ in human volunteers resulted in a spectrum of all possible labeled $PteGlu_n$ products and the progressive accumulation of $Pte[^{14}C]Glu$ (5). Incubating purified brush-border pteroylpolyglutamate hydrolase with this compound yielded the same spectrum of hydrolytic products *in vitro* (Fig. 4). The differences in times required for the appearance of labeled PteGlu, 16 h *in vitro versus* approximately 0.5 h *in vivo*, can be accounted for by the small amount of purified enzyme present in the *in vitro* incubation compared to the larger enzyme mass present in the intact surface of perfused jejunum. Significantly, *in vivo* hydrolysis of $Pte[^{14}C]GluGlu_6$ occurred during human jejunal perfusion at an intraluminal pH near 6.0, which is close to the pH at which brush-border pteroylpolyglutamate hydrolase exhibits optimal activity (Fig. 2). In addition, salicylazosulfapyridine, an anti-inflammatory drug associated with clinical folate deficiency, is a competitive inhibitor of partially purified brush-border pteroylpolyglutamate hydrolase (14) and also inhibits the *in vivo* hydrolysis of perfused $Pte[^{14}C]GluGlu_6$ (23). The availability of dietary $PteGlu_n$, but not PteGlu, is significantly decreased in human volunteers fed zinc-depleted diets (24). These observations, together with the present data on the zinc activation of purified pteroylpolyglutamate hydrolase (Fig. 6), suggest that both the activity of the brush-border enzyme and the availability of dietary pteroylpolyglutamates are influenced by intestinal zinc levels. It is less likely that intracellular mucosa pteroylpolyglutamate hydrolase plays an initial role in folate digestion since its lower pH optimum and endopeptidase activity (19) are inconsistent with observations of the *in vivo* hydrolysis of $PteGlu_n$ in humans (5). Whether both enzymes are required for the absorption of dietary folates or whether intracellular pteroylpolyglutamate hydrolase serves other mucosal metabolic functions remains unclear.

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SUPPLEMENTAL MATERIAL TO

PTEROYLPOLYGLUTAMATE HYDROLASE FROM HUMAN JEJUNAL BRUSH BORDERS:

PURIFICATION AND CHARACTERIZATION

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EXPERIMENTAL PROCEDURES

Source of tissue. Human jejunal mucosa was obtained from patients undergoing elective jejunoileal bypass surgery for obesity or revision of this operation. The amount of jejunum resected at each operation did not exceed that required for the surgical procedure. After washing the jejunal segment in ice-cold saline in the operating room, the mucosa was rapidly dissected away, wrapped in parafilm, and frozen with dry ice. Storage at −70° C for periods up to five years did not alter enzyme activity.

Chemicals and substrates. BioGel A1.5m and AG1-X8 were products of BioRad Laboratories. Molecular weight standard proteins and Sepharose CL-4B were obtained from Pharmacia. DEAE-cellulose, bile acids, and folic acid derivatives were obtained from Sigma. [^{3}H]PteGlu and [^{14}C]Glu were purchased from Amersham. $PteGlu_2$[^{14}C]Glu. Pte[^{14}C]Glu_2Glu_2. $PteGlu_3$, $PteGlu_7$, and $PteGlu_2$[^{14}C]Glu_{Leu_2} were synthesized by a solid phase method (6) and were provided by Dr. Carlos Krumdieck, University of Alabama. All other chemicals used were the purest available from commercial sources.

PURIFICATION OF HUMAN BORDER PPH

Enzyme Assay. The activity of brush border PPH was measured by a modification of the charcoal adsorption method (7). The standard reaction mixture consisted of 13 μM $PteGlu_2$[^{14}C]Glu, 66 μM glutamic acid, 0.1 mM zinc acetate, and 0.27 M NaCl in 33 mM 3,3-dimethylglutarate at pH 6.5 and enzyme to a final volume of 0.75 ml. Mucosal homogenates were assayed in the presence of 0.17 mM p-hydroxymercuribenzoate, a compound which completely inhibits intracellular PPH but which has no effect on brush border PPH (4). After incubation for 30 minutes at 37° C, reactions were terminated by adding 0.25 ml of 10% trichloroacetic acid. Unreacted substrate was adsorbed with 0.5 ml of 2% charcoal in 0.1 M acetic acid. After centrifugation, [^{14}C]Glu in the supernate was quantitated by liquid scintillation counting. Preliminary studies using [^{3}H]PteGlu or [^{14}C]Glu in appropriate concentrations showed that the assay conditions resulted in complete adsorption of the pteroyl moiety with quantitative recovery of [^{14}C]Glu in the supernatant. The presence of detergents such as Triton X-100 or bile acids in the concentrations employed did not affect charcoal binding. For all initial velocity and inhibition studies, less than 10% of the substrate was hydrolyzed during the reaction period. Protein concentrations were measured by the Bradford method (8). Enzyme activity is expressed as milliUnits (mUnits) per mg protein where one mUnit is defined as 1 nmole products liberated per minute.

Brush border membrane isolation and solubilization. Approximately 30 g of minced jejunal mucosa were homogenized in 50 mM Tris-Cl buffer, pH 7.0 (10% weight/volume), using a Brinkman polytron homogenizer, followed by vacuum filtration through cheesecloth. The brush border fractions were prepared by a modification of the method of Schmitz et al (9). Following centrifugation of the homogenate at 30,000 X g for 15 minutes, the pellet was suspended in 450 ml of 2 mM Tris and 50 mM mannitol, pH 7.0. This was followed by the addition of 50 ml of 100 mM $CaCl_2$ in the same buffer. After stirring at 4° C for 30 minutes, the mixture was centrifuged at 2000 X g for 10 minutes, and the resulting supernate was centrifuged at 30,000 X g for 30 minutes. The pellet was washed in 50 ml of the same buffer and recentrifuged at 30,000 X g for 30 minutes. The specific activity of sucrase (18) in the brush border pellet was enriched 15-fold, compared to the homogenate. The pellet was then solubilized in 20 ml of buffer containing 0.5% Triton X-100, 10 mM Tris-Cl, 0.6 M NaCl, 0.1 mM zinc acetate, and 2 mM 2-mercaptoethanol at pH 7.0. After stirring at 4° C for 30 minutes and centrifugation at 30,000 X g for 30 minutes, PPH was recovered in the final supernate.

Organomercurial sepharose chromatography. A p-aminophenylmercury sepharose column was constructed (11) in order to separate the two intestinal enzymes by adsorption of intracellular PPH and to achieve further purification of brush border PPH. The column (1 X 25 cm) was equilibrated with buffer containing 10 mM Tris-Cl, 0.4 M NaCl, 0.1 mM zinc acetate, 2 mM 2-mercaptoethanol, and 0.1% Triton X-100 at pH 7.0. After application of the Triton-solubilized supernate to the column, brush border PPH appeared between 30 and 45 ml of eluate and was pooled. This step resulted in the complete exclusion of intracellular PPH, as shown by absence of inhibition of the eluted PPH activity by p-hydroxymercuribenzoate.

DEAE-cellulose chromatography. A DEAE-cellulose column (1.5 X 25 cm) was equilibrated with buffer containing 10 mM Tris-Cl, 0.1 mM zinc acetate, 2 mM 2-mercaptoethanol, and 0.1% Triton X-100 at pH 8.5, hereafter referred to as standard buffer. After dialysis against 3 one liter volumes of buffer, the organomercurial column pool was applied to the DEAE-cellulose column and washed with 100 ml of buffer. Using a 400 ml gradient of 0 to 0.2 M NaCl in standard buffer, PPH was eluted at a rate of 1 ml per minute and appeared between 50 and 100 ml of the gradient.

Gel filtration and molecular weight determination. The DEAE-cellulose column pool was dialyzed against buffer as described above, followed by concentration on a 2 ml column of DEAE-cellulose and elution with 0.5 M NaCl in standard buffer. The resulting 3 to 4 ml sample was applied to a BioGel A1.5m column (2 X 100 cm), which was equilibrated with buffer containing 0.1 M NaCl. Two ml fractions were collected with a flow rate of 10 ml/hr. The proteins used as molecular weight standards were thyroglobulin (660,000), ferritin (440,000), catalase (235,000), aldolase (158,000), and bovine serum albumin (67,000). Blue Dextran 2000 and [^{14}C]Glu were used for void volume and total volume markers, respectively.

CHARACTERIZATION OF HUMAN BORDER PPH

Samples of the purified enzyme (6 μg) were analyzed by SDS-urea PAGE (12) using 6% polyacrylamide slab gels. The gels were stained with 0.05% Coomassie brilliant blue R250 in 10% acetic acid and 50% methanol or silver stained (13). The isoelectric point of PPH in the DEAE-cellulose pool was determined with an LKB isoelectric focusing column (110 ml) using a 0 to 40% sucrose gradient and 2% ampholyte, pH 4 to 9. After equilibration for 24 hr at 4 mAmp, the column was eluted and 2 ml fractions were collected. The pH and temperature dependence of PPH activity and stability were measured over a range of conditions.

The mechanism of brush border PPH hydrolysis was studied by chromatographic identification of the glutamate products of $PteGlu_2[^{14}C]Glu$ hydrolysis and of the labeled products of $Pte[^{14}C]Glu_6$ hydrolysis. To determine whether PPH is capable of cleaving internal linkages, its activity was measured using the standard assay and substituting $PteGlu_2[^{14}C]Glu_2$ (1.5 and 50 μM) as substrate. The kinetic constants of hydrolysis were determined by measuring initial velocities at varied concentrations of $PteGlu_2[^{14}C]Glu$. The effects of glutamate chain length on PPH activity were studied by measuring the initial velocity of $PteGlu_2[^{14}C]Glu$ hydrolysis in the presence of different fixed concentrations of PteGlu (0 to 64 μM), $PteGlu_2$ (0 to 10 μM), and $PteGlu_7$ (0 to 12 μM). We also studied the effects of various moieties of $PteGlu_n$ on brush border PPH activity. Enzyme activity was measured at 4 μM of $PteGlu_2[^{14}C]Glu$ and 0.1 and 1.0 mM of PteGlu, 5-CH_3-H_4PteGlu, 5-CHO-H_4PteGlu, Glu, γ-Glu_2, α-Glu_2, α-Glu_3, p-aminobenzoate, N-benzoylglutamate, N-p-aminobenzoylglutamate, N-p-nitrobenzoylglutamate, or pterin carboxylate.

The effects of various cations on PPH activity were studied using enzyme dialyzed against 100 ml of standard buffer minus zinc, containing 1 mM phenanthroline and 1 mM EDTA. After extensive dialysis to remove the chelators, standard assays were performed with the addition of 0.02, 0.10, and 1.0 mM concentrations of the chloride salts of Zn^{+2}, Co^{+2}, Ni^{+2}, Fe^{+2}, Co^{+2}, Cd^{+2}, Mn^{+2}, Mg^{+2}, Pb^{+2}, Cu^{+2}, and Hg^{+2}. Glutamic acid was omitted from the reaction mixtures to avoid potential chelation of the added metals. The effects of different anions on PPH activity were studied using enzyme dialized against standard buffer minus zinc. PPH activity was assayed with the addition of varied concentrations (0.1 to 0.3 M) of NaCl, KCl, NaBr, NaF; $NaNO_3$ and Na-1,3-diethylglutarate in the absence of added Zn^{+2}. To confirm the previous observation that p-hydroxymercuribenzoate is not inhibitory, the effect of various concentrations of this compound (0.05 to 0.5 mM) on the activity of the purified enzyme was studied. The effects of cholate, deoxycholate and chenodeoxycholate and their glycine and taurine conjugates on PPH activity were studied using non-solubilized brush border membranes in the standard reaction mixture with 4 μM $PteGlu_2[^{14}C]Glu$. The different bile acids were added at concentrations of 0.01, 0.1 and 1.0 mM.